

ALTERATION OF AMINO ACID COMPOSITIONS IN SEEDS

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BACKGROUND OF THE INVENTION

Feed formulations based on crop plants must typically be supplemented with specific amino acids to provide animals with essential nutrients which are necessary for their growth. This supplementation is necessary because, in general, crop plants contain low proportions of several amino acids which are essential for, and cannot be synthesized by, monogastric animals.

The seeds of crop plants contain different classes of seed proteins. The amino acid composition of these seeds reflects the composition of the prevalent classes of proteins. Amino acid limitations are usually due to amino acid deficiencies of these prevalent protein classes.

Among the amino acids necessary for animal nutrition, those that are of limited availability in crop plants include methionine, lysine, and threonine. Attempts to increase the levels of these amino acids by breeding, mutant selection, and/or changing the composition of the storage proteins accumulated in the seeds of crop plants, have met with limited success, or were accompanied by a loss in yield.

For example, although seeds of corn plants containing a mutant transcription factor, (opaque 2), or a mutant α -zein gene, (floury 2), exhibit elevated levels of total and bound lysine, there is an altered seed endosperm structure which is more susceptible to damage and pests. Significant yield losses are also typical.

An alternative means to enhance levels of free amino acids in a crop plant is the modification of amino acid biosynthesis in the plant. The introduction of a feedback-regulation-insensitive dihydrodipicolinic acid synthase ("DHDPS") gene, which encodes an enzyme that catalyzes the first reaction unique to the lysine biosynthetic pathway, into plants has resulted in an increase in the levels of free lysine in the leaves and seeds of those plants. An increase in the levels of free lysine in the embryo results in reduced amount of oil in the seed. Further free lysine can be lost during the wet milling process reducing the feed value of the gluten product of the process.

The expression of the *lysC* gene, which encodes a mutant bacterial aspartate kinase that is desensitized to feedback inhibition by lysine and threonine, from a seed-specific promoter in tobacco plants, has resulted in an increase in methionine and threonine biosynthesis in the seeds of those plants. See Karchi, *et al.*; The Plant J.; Vol. 3; p. 721; (1993). However, expression of the *lysC* gene results in only a 6-7% increase in the level of total threonine or methionine in the seed. The expression of the *lysC* gene in seeds has a minimal impact on the nutritional value of those seeds and, thus, supplementation of feed containing *lysC* transgenic seeds with amino acids, such as methionine and threonine, is still required.

There are additional molecular genetic strategies available for enhancing the amino acid quality of plant proteins. Each involves molecular manipulation of plant genes and the generation of transgenic plants.

Protein sequence modification involves the identification of a gene encoding a major protein, preferably a storage protein, as the target for modification to contain more codons of essential amino acids. An important aspect of this approach is to be

able to select a region of the protein that can be modified without affecting the overall structure, stability, function, and other cellular and nutritional properties of the protein.

The development of DNA synthesis technology allows the design and
5 synthesis of a gene encoding a new protein with desirable essential amino acid compositions. For example, researchers have synthesized a 292-base pair DNA sequence encoding a polypeptide composed of 80% essential amino acids and used it with the nopaline synthetase (NOS) promoter to construct a chimeric gene. Expression of this gene in the tuber of transgenic potato has resulted in an
10 accumulation of this protein at a level of 0.02% to 0.35% of the total plant protein. This low level accumulation is possibly due to the weak NOS promoter and/or the instability of the new protein.

Tobacco has been used as a test plant to demonstrate the feasibility of this approach by transferring a chimeric gene containing the bean phaseolin promoter
15 and the cDNA of a sulfur-rich protein Brazil Nut Protein ("BNP"), (18 mol% methionine and 8 mol% cysteine) into tobacco. Amino acid analysis indicates that the methionine content in the transgenic seeds is enhanced by 30% over that of the untransformed seeds. This same chimeric gene has also been transferred into a commercial crop, canola, and similar levels of enhancement were achieved.

20 However, an adverse effect is that lysine content decreases. Additionally, BNP has been identified as a major food allergen. Thus it is neither practical nor desirable to use BNP to enhance the nutritional value of crop plants.

Thus, there is a need to improve the nutritional value of plant seeds. The genetic modification should not be accompanied by detrimental side effects such as allergenicity, anti-nutritional quality or poor yield.

SUMMARY OF THE INVENTION

5 It is an object of the present invention to provide a seed, the endosperm of which contains elevated levels of an essential amino acid.

It is a further object of the present invention to provide methods for increasing the nutritional value of feed.

10 It is a further object of the present invention to provide methods for genetically modifying seeds so as to increase amounts of essential amino acids which are present in relatively low amounts in unmodified seeds.

It is a further object of the present invention to provide methods for increasing the nutritional content of seeds without detrimental side effects such as allergenicity or anti-nutritional quality.

15 It is a further object of the present invention to provide methods for increasing the nutritional content of seeds while maintaining a high yield.

It is a further object of the present invention to provide a method for the expression of a polypeptide in a seed having levels of a preselected amino acid sufficient to reduce or obviate feed supplementation.

20 According to the present invention a transformed plant seed is provided, the endosperm of which is characterized as having an elevated level of at least one preselected amino acid compared to a seed from a corresponding plant which has not been transformed, wherein the amino acid is lysine, threonine, or tryptophan and optionally a sulfur-containing amino acid.

Also provided is a seed from a plant which has been transformed to express a heterologous protein in the endosperm of the seed, wherein the seed exhibits an elevated level of an essential amino acid.

5 An expression cassette is also provided comprising a seed endosperm-preferred promoter operably linked to a structural gene encoding a polypeptide having an elevated level of a preselected amino acid. Transformed plants and seeds containing the expression cassette are also provided.

10 A method for elevating the level of a preselected amino acid in the endosperm of plant seed is also provided. The method comprises the transformation of plant cells by introducing the expression cassette, recovering the transformed cells, regenerating a transformed plant and collecting the seeds therefrom.

DETAILED DESCRIPTION OF THE INVENTION

15 As used herein, a "structural gene " means an exogenous or recombinant DNA sequence or segment that encodes a polypeptide.

As used herein, "recombinant DNA" is a DNA sequence or segment that has been isolated from a cell, purified, synthesized or amplified.

As used herein, "isolated" means either physically isolated from the cell or synthesized *in vitro* on the basis of the sequence of an isolated DNA segment.

20 As used herein, the term "increased" or "elevated" levels of the preselected amino acid in a protein means that the protein contains an elevated amount of a preselected amino acid compared to the amount in an average protein.

As used herein, "increased" or "elevated" levels or amounts of preselected amino acids in a transformed plant or seed are levels which are greater than the levels or amounts in the corresponding untransformed plant or seed.

As used herein, "polypeptide" means proteins, protein fragments, modified
5 proteins, amino acid sequences and synthetic amino acid sequences.

As used herein, "transformed plant" means a plant which comprises a structural gene which is introduced into the genome of the plant by transformation.

As used herein, "untransformed plant" refers to a wild type plant, i.e., one where the genome has not been altered by the introduction of the structural gene.

10 As used herein, "plant" includes but is not limited to plant cells, plant tissue and plant seeds.

As used herein, "seed endosperm-preferred promoter" is a promoter which preferentially promotes expression of the structural gene in the endosperm of the seed.

15 As used herein with respect to a structural gene encoding a polypeptide, the term "expresses" means that the structural gene is incorporated into the genome of cells, so that the product encoded by the structural gene is produced within the cells.

As used herein, the term "essential amino acid" means an amino acid which is synthesized only by plants or microorganisms or which is not produced by animals
20 in sufficient quantities to support normal growth and development.

As used herein, the term "high lysine content protein" means that the protein has at least about 7 mole % lysine, preferably about 7 mole % to about 50 mole % lysine, more preferably about 7 mole % to about 40 mole % lysine and most preferably about 7 mole % to about 30 mole %.

As used herein, the term "high sulfur content protein" means that the protein contains at least about 6 mole % methionine and/or cysteine, preferably about 6 mole % to about 40 mole %, more preferably about 6 mole % to about 30 mole % and most preferably 6 mole % to 25 mole %.

5 The present invention provides a transformed plant seed, the endosperm of which is characterized as having an elevated level of a preselected amino acid compared to the seed of a corresponding plant which has not been transformed. It is preferred that the level of preselected amino acid is elevated in the endosperm in preference to other parts of the seed.

10 The preselected amino acid is an essential amino acid such as lysine, cysteine, methionine, threonine, tryptophan, arginine, valine, leucine, isoleucine, histidine or combinations thereof, preferably, the preselected amino acid is lysine, threonine, cysteine, tryptophan, or combinations thereof and optionally methionine. It is especially preferred that the polypeptide has an increased content of lysine as
15 well as a sulfur containing amino acid, i.e., methionine and/or cysteine.

 The polypeptide can be an endogenous or heterologous protein. When an endogenous protein is expressed, the preselected amino acid is lysine, cysteine, threonine, tryptophan, arginine, valine, leucine, isoleucine, histidine or combinations thereof and optionally methionine. When the protein is a heterologous protein, any
20 of the above described preselected amino acids or combinations thereof is present in elevated amounts.

 Generally the amount of preselected amino acid in the seed of the present invention is at least about 10 percent by weight greater than in a corresponding untransformed seed, preferably about 10 percent by weight to about 10 times

greater, more preferably about 15 percent by weight to about 10 time greater and most preferably about 20 percent to about 10 times greater.

A polypeptide having an elevated amount of the preselected amino acid is expressed in the transformed plant seed endosperm in an amount sufficient to
5 increase the amount of at least one preselected amino acid in the seed of the transformed plant, relative to the amount of the preselected amino acid in the seed of a corresponding untransformed plant.

The choice of the structural gene is based on the desired amino acid composition of the polypeptide encoded by the structural gene, and the ability of the
10 polypeptide to accumulate in seeds. The amino acid composition of the polypeptide can be manipulated by methods, such as site-directed mutagenesis of the structural gene encoding the polypeptide, so as to result in expression of a polypeptide that is increased in the amount of a particular amino acid. For example, site-directed
15 mutagenesis can be used to increase levels of lysine, methionine, cysteine, threonine and/or tryptophan and/or to decrease levels of asparagine and/or glutamine.

The derivatives differ from the wild-type protein by one or more amino acid substitutions, insertions, deletions or the like. Typically, amino acid substitutions are conservative. In the regions of homology to the native sequence, variants preferably
20 have at least 90% amino acid sequence identity, more preferably at least 95% identity.

Typical examples of suitable proteins include barley chymotrypsin inhibitor, barley alpha hordothionin, soybean 2S albumin proteins, ~~rice high methionine~~
protein and sunflower high methionine protein and derivatives of each protein.

Barley alpha hordothionin has been modified to increase the level of particular amino acids. The sequences of genes which express modified alpha hordothionin proteins with enhanced essential amino acids are based on the mRNA sequence of the native *Hordeum vulgare* alpha hordothionin gene (accession number X05901, Ponz *et al.* 1986 Eur. J. Biochem. 156:131-135).

~~Modified hordothionin proteins are described in U.S. Ser. Nos. 08/838,763 filed April 10, 1997; 08/824,379 filed March 26, 1997; 08/824,382 filed March 26, 1997; and U.S. Pat. No. 5,703,409 issued December 30, 1997 the disclosures of which are incorporated herein in their entirety by reference.~~

Alpha hordothionin is a 45-amino acid protein which is stabilized by four disulfide bonds resulting from eight cysteine residues. In its native form, the protein is especially rich in arginine and lysine residues, containing 5 residues (10%) of each. However, it is devoid of the essential amino acid methionine.

Alpha hordothionin has been modified to increase the amount of various amino acids such as lysine, threonine or methionine. The protein has been synthesized and the three-dimensional structure determined by computer modeling. The modeling of the protein predicts that the ten charged residues (arginine at positions 5, 10, 17, 19 and 30, and lysine at positions 1, 23, 32, 38 and 45) all occur on the surface of the molecule. The side chains of the polar amino acids (asparagine at position 11, glutamine at position 22 and threonine at position 41) also occur on the surface of the molecule. Furthermore, the hydrophobic amino acids, (such as the side chains of leucine at positions 8, 15, 24 and 33 and valine at position 18) are also solvent-accessible.

The Three-dimensional modeling of the protein indicates that the arginine residue at position 10 is important to retention of the appropriate 3-dimensional structure and possible folding through hydrogen bond interactions with the C-terminal residue of the protein. A lysine, methionine or threonine substitution at that point would disrupt this hydrogen bonding network, leading to a destabilization of the structure. The synthetic peptide having this substitution could not be made to fold correctly, which supported this analysis. Conservation of the arginine residue at position 10 provides a protein which folds correctly.

Alpha hordothionin has been modified to contain 12 lysine residues in the mature hordothionin peptide, referred to as HT12. (Rao *et al.* 1994 Protein Engineering 7(12):1485-1493 and WO 94/16078 published July 21, 1994) The disclosure of each of these is incorporated herein by reference in their entirety.

Further analysis of substitutions which would not alter the 3-dimensional structure of the molecule led to replacement of Asparagine-11, Glutamine-22 and Threonine-41 with lysine residues with virtually no steric hindrance. The resulting compound contains 27% lysine residues.

Other combinations of these substitutions were also made, including changes in amino acid residues at one or more of positions 5, 11, 17, 19, 22, 30 and 41 are lysine, and the remainder of the residues at those positions are the residues at the corresponding positions in the wild type hordothionin.

Since threonine is a polar amino acid, the surface polar amino acid residues, asparagine at position 11 and glutamine at position 22, can be substituted; and the charged amino acids, lysine at positions 1, 23, 32 and 38 and arginine at positions 5,

17, 19, and 30, can also be substituted with threonine. The molecule can be synthesized by solid phase peptide synthesis.

While the above sequence is illustrative of the present invention, it is not intended to be a limitation. Threonine substitutions can also be performed at positions containing charged amino acids. Only arginine at position 10 and lysine at position 45 are important for maintaining the structure of the protein. One can also substitute at the sites having hydrophobic amino acids. These include positions 8, 15, 18 and 24.

Since methionine is a hydrophobic amino acid, the surface hydrophobic amino acid residues, leucine at positions 8, 15, and 33, and valine at position 18, were substituted with methionine. The surface polar amino acids, asparagine at position 11, glutamine at position 22 and threonine at position 41, are substituted with methionine. The molecule is synthesized by solid phase peptide synthesis and folds into a stable structure. It has seven methionine residues (15.5%) and, including the eight cysteines, the modified protein has a sulfur amino acid content of 33%.

While the above-described proteins are illustrative of suitable polypeptides which can be expressed in the transformed plant, it is not intended to be a limitation. Methionine substitutions can also be performed at positions containing charged amino acids. Only arginine at position 10 is important for maintaining the structure of the protein through a hydrogen-bonding network with serine at position 2 and lysine at position 45. Thus, one can substitute methionine for lysine at positions 1, 23, 32, and/or 38, and for arginine at positions 5, 17, 19 and/or 30.

Many other proteins are also appropriate, for example the protein encoded by the structural gene can be a lysine and/or sulfur rich seed protein, such as the

soybean 2S albumin described in U.S. Ser. No. 08/618,911 filed March 20, 1996,

and the chymotrypsin inhibitor from barley, Williamson et al., Eur. J Biochem 165:

99-106 (1987), the disclosures of each are incorporated by reference.

Derivatives of these genes can be made by site directed mutagenesis to increase the level of preselected amino acids in the encoded polypeptide. For

example the gene encoding for the barley high lysine polypeptide (BHL), is derived from barley chymotrypsin inhibitor, U.S. Ser. No. 08/740,682 filed November 1, 1996

and PCT/US97/20441 filed October 31, 1997, the disclosures of each are incorporated herein by reference. The gene encoding for the enhanced soybean

albumin gene (ESA), is derived from soybean 2S albumin described in U.S. Ser. No. 08/618,911, the disclosure of which is incorporated herein in its entirety by reference.

Other examples of sulfur-rich plant proteins within the scope of the invention include plant proteins enriched in cysteine but not methionine, such as the wheat

endosperm purothionine (Mak and Jones; Can. J. Biochem.; Vol. 22; p. 83J; (1976);

incorporated herein in its entirety by reference), the pea low molecular weight albumins (Higgins, et al.; J. Biol. Chem.; Vol. 261; p. 11124; (1986); incorporated

herein in its entirety by reference) as well as 2S albumin genes from other organisms. See, for example, Coulter, et al.; J. Exp. Bot.; Vol. 41; p. 1541; (1990);

incorporated herein in its entirety by reference.

Such proteins also include methionine-rich plant proteins ~~such as from~~
~~sunflower seed~~ (Lilley, *et al.*; In: Proceedings of the World Congress on Vegetable
Protein Utilization in Human Foods and Animal Feedstuffs; Applewhite, H. (ed.);
American Oil Chemists Soc.; Champaign, IL; pp. 497-502; (1989); incorporated
5 herein in its entirety by reference), corn (Pedersen, *et al.*; J. Biol. Chem. p. 261; p.
6279; (1986); Kirihaara, *et al.*; Gene, Vol. 71; p. 359; (1988); both incorporated herein
in its entirety by reference), and rice (Musumura, *et al.*; Plant Mol. Biol.; Vol. 12; p.
123; (1989); incorporated herein in its entirety by reference).

The present invention also provides a method for genetically modifying plants
10 to increase the level of at least one preselected amino acid in the endosperm of the
seed so as to enhance the nutritional value of the seeds.

The method comprises the introduction of an expression cassette into
regenerable plant cells to yield transformed plant cells. The expression cassette
comprises a seed endosperm-preferred promoter operably linked to a structural
15 gene encoding a polypeptide elevated in content of the preselected amino acid.

A fertile transformed plant is regenerated from the transformed cells, and
seeds are isolated from the plant. The structural gene is transmitted through a
complete normal sexual cycle of the transformed plant to the next generation.

The polypeptide is synthesized in the endosperm of seed of the plant which
20 has been transformed by insertion of the expression cassette described above.
The sequence for the nucleotide molecule, either RNA or DNA, can readily be
derived from the amino acid sequence for the selected polypeptide using standard
reference texts.

Plants which can be used in the method of the invention include monocotyledonous cereal plants. Preferred plants include maize, wheat, rice, barley, oats, sorghum, millet and rye. The most preferred plant is maize.

Seeds derived from plants regenerated from transformed plant cells, plant parts or plant tissues, or progeny derived from the regenerated transformed plants, may be used directly as feed or food, or further processing may occur.

Transformation

The transformation of plants in accordance with the invention may be carried out in essentially any of the various ways known to those skilled in the art of plant molecular biology. These include, but are not limited to, microprojectile bombardment, microinjection, electroporation of protoplasts or cells comprising partial cell walls, and *Agrobacterium*-mediated DNA transfer.

I. DNA Used for Transformation

DNA useful for introduction into plant cells includes DNA that has been derived or isolated from any source, that may be subsequently characterized as to structure, size and/or function, chemically altered, and later introduced into the plant.

An example of DNA "derived" from a source, would be a DNA sequence or segment that is identified as a useful fragment within a given organism, and which is then synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from the source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering.

Therefore, useful DNA includes completely synthetic DNA, semi-synthetic DNA, DNA isolated from biological sources, and DNA derived from RNA. The DNA isolated from biological sources, or DNA derived from RNA, includes, but is not limited to, DNA or RNA from plant genes, and non-plant genes such as those from bacteria, yeasts, animals or viruses. The DNA or RNA can include modified genes, portions of genes, or chimeric genes, including genes from the same or different genotype.

The term "chimeric gene" or "chimeric DNA" is defined as a gene or DNA sequence or segment comprising at least two DNA sequences or segments from species which do not recombine DNA under natural conditions, or which DNA sequences or segments are positioned or linked in a manner which does not normally occur in the native genome of untransformed plant.

A structural gene of the invention can be identified by standard methods, e.g., enrichment protocols, or probes, directed to the isolation of particular nucleotide or amino acid sequences. The structural gene can be identified by obtaining and/or screening of a DNA or cDNA library generated from nucleic acid derived from a particular cell type, cell line, primary cells, or tissue.

Screening for DNA fragments that encode all or a portion of the structural gene can be accomplished by screening plaques from a genomic or cDNA library for hybridization to a probe of the structural gene from other organisms or by screening plaques from a cDNA expression library for binding to antibodies that specifically recognize the polypeptide encoded by the structural gene.

DNA fragments that hybridize to a structural gene probe from other organisms and/or plaques carrying DNA fragments that are immunoreactive with antibodies to the polypeptide encoded by the structural gene can be subcloned into a vector and sequenced and/or used as probes to identify other cDNA or genomic sequences encoding all or a portion of the structural gene.

Portions of the genomic copy or copies of the structural gene can be partially sequenced and identified by standard methods including either DNA sequence homology to other homologous genes or by comparison of encoded amino acid sequences to known polypeptide sequences.

Once portions of the structural gene are identified, complete copies of the structural gene can be obtained by standard methods, including cloning or polymerase chain reaction (PCR) synthesis using oligonucleotide primers complementary to the structural gene. The presence of an isolated full-length copy of the structural gene can be verified by comparison of its deduced amino acid sequence with the amino acid sequence of native polypeptide sequences.

As discussed above, the structural gene encoding the polypeptide can be modified to increase the content of particular amino acid residues in that polypeptide by methods well known to the art, including, but not limited to, site-directed mutagenesis. Thus, derivatives of naturally occurring polypeptides can be made by nucleotide substitution of the structural gene so as to result in a polypeptide having a different amino acid at the position in the polypeptide which corresponds to the codon with the nucleotide substitution. The introduction of multiple amino acid changes in a polypeptide can result in a polypeptide which is significantly enriched in a preselected amino acid.

As noted above, the choice of the polypeptide encoded by the structural gene will be based on the amino acid composition of the polypeptide and its ability to accumulate in seeds. The amino acid can be chosen for its nutritional value to produce a value-added trait to the plant or plant part. Amino acids desirable for value-added traits, as well as a source to limit synthesis of an endogenous protein include, but are not limited to, lysine, threonine, tryptophan, methionine, and cysteine.

Expression Cassettes and Expression Vectors

According to the present invention, a structural gene is identified, isolated, and combined with a seed endosperm-preferred promoter to provide a recombinant expression cassette.

The construction of such expression cassettes which can be employed in conjunction with the present invention are well known to those of skill in the art in light of the present disclosure. See, e.g., Sambrook, *et al.*; Molecular Cloning: A Laboratory Manual; Cold Spring Harbor, New York; (1989); Gelvin, *et al.*; Plant Molecular Biology Manual; (1990); Plant Biotechnology: Commercial Prospects and Problems, eds Prakash, *et al.*; Oxford & IBH Publishing Co.; New Delhi, India; (1993); and Heslot, *et al.*; Molecular Biology and Genetic Engineering of Yeasts; CRC Press, Inc., USA; (1992); each incorporated herein in its entirety by reference.

Preferred promoters useful in the practice of the invention are those seed endosperm-preferred promoters that allow expression of the structural gene selectively in seed endosperm to avoid any potential deleterious effects associated with the expression of the structural gene in the embryo.

It has been found that when endosperm-preferred promoters are employed, the total level of the preselected amino acid in the seed is increased compared to a seed produced by employing an embryo-preferred promoter, such as the globulin1 promoter. When the globulin1 promoter is employed, the polypeptide is expressed by the structural gene, but the total amount of the preselected amino acid is not increased.

Examples of suitable promoters include, but are not limited to, 27 kD gamma zein promoter and waxy promoter. See the following sites relating to the 27kD gamma zein promoter: Boronat,A., Martinez,M.C., Reina,M., Puigdomenech,P. and Palau,J.; Isolation and sequencing of a 28 kD glutelin-2 gene from maize: Common elements in the 5' flanking regions among zein and glutelin genes; Plant Sci. 47, 95-102 (1986) and Reina,M., Ponte,I., Guillen,P., Boronat,A. and Palau,J., Sequence analysis of a genomic clone encoding a Zc2 protein from Zea mays W64 A, Nucleic Acids Res. 18 (21), 6426 (1990). See the following site relating to the waxy promoter: Kloesgen,R.B., Gierl,A., Schwarz-Sommer,ZS. and Saedler,H., Molecular analysis of the waxy locus of Zea mays, Mol. Gen. Genet. 203, 237-244 (1986). The disclosures each of these are incorporated herein by reference in their entirety.

However, other endosperm-preferred promoters can be employed.

II. DELIVERY OF DNA TO CELLS

The expression cassette or vector can be introduced into prokaryotic or eukaryotic cells by currently available methods which are described in the literature. See for example, Weising *et al.*, Ann. Rev. Genet. 2: 421-477 (1988). For example, the expression cassette or vector can be introduced into plant cells by methods including, but not limited to, *Agrobacterium*-mediated transformation, electroporation,

PEG poration, microprojectile bombardment, microinjection of plant cell protoplasts or embryogenic callus, silicon fiber delivery, infectious viruses or viroids such as retroviruses, the use of liposomes and the like, all in accordance with well-known procedures.

5 The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski *et al.*, Embo J. 3: 2717-2722 (1984). Electroporation techniques are described in Fromm *et al.*, Proc. Natl. Acad. Sci. 82: 5324 (1985). Ballistic transformation techniques are described in Klein *et al.*, Nature 327: 70-73 (1987). The disclosure of each of these is incorporated herein in its entirety by
10 reference.

 Introduction and expression of foreign genes in plants has been shown to be possible using the T-DNA of the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens*. Using recombinant DNA techniques and bacterial genetics, a wide variety of foreign DNAs can be inserted into T-DNA in *Agrobacterium*. Following
15 infection by the bacterium containing the recombinant Ti plasmid, the foreign DNA is inserted into the host plant chromosomes, thus producing a genetically engineered cell and eventually a genetically engineered plant. A second approach is to introduce root-inducing (Ri) plasmids as the gene vectors.

Agrobacterium tumefaciens-mediated transformation techniques are well
20 described in the literature. See, for example Horsch *et al.*, Science 233: 496-498 (1984), and Fraley *et al.*, Proc. Natl. Acad. Sci. 80: 4803 (1983). *Agrobacterium* transformation of maize is described in U.S. Patent No. 5,550,318. The disclosure of each of these is incorporated herein in its entirety by reference.

Other methods of transfection or transformation include (1) *Agrobacterium rhizogenes*-mediated transformation (see, e.g., Lichtenstein and Fuller In: Genetic Engineering, vol. 6, PWJ Rigby, Ed., London, Academic Press, 1987; and Lichtenstein, C. P., and Draper, J., In: DNA Cloning, Vol. II, D. M. Glover, Ed., Oxford, IRI Press, 1985). Application PCT/US87/02512 (WO 88/02405 published Apr. 7, 1988) describes the use of *A. rhizogenes* strain A4 and its Ri plasmid along with *A. tumefaciens* vectors pARC8 or pARC16 (2) liposome-mediated DNA uptake (see, e.g., Freeman *et al.*, Plant Cell Physiol. 25: 1353, 1984), (3) the vortexing method (see, e.g., Kindle, Proc. Natl. Acad. Sci., USA 87: 1228, (1990). The disclosure of each of these is incorporated herein in its entirety by reference.

DNA can also be introduced into plants by direct DNA transfer into pollen as described by Zhou *et al.*, Methods in Enzymology, 101:433 (1983); D. Hess, Intern Rev. Cytol., 107:367 (1987); Luo *et al.*, Plane Mol. Biol. Reporter, 6:165 (1988). The disclosure of each of these is incorporated herein in its entirety by reference.

Expression of polypeptide coding genes can be obtained by injection of the DNA into reproductive organs of a plant as described by Pena *et al.*, Nature, 325:274 (1987). The disclosure of which is incorporated herein in its entirety by reference.

DNA can also be injected directly into the cells of immature embryos and the rehydration of desiccated embryos as described by Neuhaus *et al.*, Theor. Appl. Genet., 75:30 (1987); and Benbrook *et al.*, in Proceedings Bio Expo 1986, Butterworth, Stoneham, Mass., pp. 27-54 (1986). The disclosure of each of these is incorporated herein in its entirety by reference.

Plant cells useful for transformation include cells cultured in suspension cultures, callus, embryos, meristem tissue, pollen, and the like.

A variety of plant viruses that can be employed as vectors are known in the art and include cauliflower mosaic virus (CaMV), geminivirus, brome mosaic virus, and tobacco mosaic virus.

Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers *et al.*, Meth. In Enzymol., 153:253-277 (1987). These vectors are plant integrating vectors in that on transformation, the vectors integrate a portion of vector DNA into the genome of the host plant. The disclosure of which is incorporated herein in its entirety by reference.

A particularly preferred vector is a plasmid, by which is meant a circular double-stranded DNA molecule which is not a part of the chromosomes of the cell. Exemplary *A. tumefaciens* vectors useful herein are plasmids pKYLX6 and pKYLX7 of Schardl *et al.*, Gene, 61:1-11 (1987) and Berger *et al.*, Proc. Natl. Acad. Sci. U.S.A., 86:8402-8406 (1989). Another useful vector herein is plasmid pBI101.2 that is available from Clontech Laboratories, Inc. (Palo Alto, CA). The disclosure of each of these is incorporated herein in its entirety by reference.

A cell in which the foreign genetic material in a vector is functionally expressed has been "transformed" by the vector and is referred to as a "transformant".

Either genomic DNA or cDNA coding the gene of interest may be used in this invention. The gene of interest may also be constructed partially from a cDNA clone and partially from a genomic clone.

When the gene of interest has been isolated, genetic constructs are made which contain the necessary regulatory sequences to provide for efficient expression of the gene in the host cell.

According to this invention, the genetic construct will contain (a) a genetic
5 sequence coding for the polypeptide of interest and (b) one or more regulatory sequences operably linked on either side of the structural gene of interest. Typically, the regulatory sequences will be a promoter or a terminator. The regulatory sequences may be from autologous or heterologous sources.

The cloning vector will typically carry a replication origin, as well as specific
10 genes that are capable of providing phenotypic selection markers in transformed host cells. Typically, genes conferring resistance to antibiotics or selected herbicides are used. After the genetic material is introduced into the target cells, successfully transformed cells and/or colonies of cells can be isolated by selection on the basis of these markers.

15 Typical selectable markers include genes coding for resistance to the antibiotic spectinomycin (e.g., the aada gene), the streptomycin phosphotransferase (SPT) gene coding for streptomycin resistance, the neomycin phosphotransferase (NPTII) gene encoding kanamycin or geneticin resistance, the hygromycin phosphotransferase (HPT) gene coding for hygromycin resistance.

20 Genes coding for resistance to herbicides include genes which act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) genes containing mutations leading to such resistance in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides which act to inhibit action of glutamine synthase, such as

phosphinothricin or basta (e.g., the *pat* or *bar* gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta, and the ALS gene encodes resistance to the herbicide chlorsulfuron.

Typically, an intermediate host cell will be used in the practice of this invention to increase the copy number of the cloning vector. With an increased copy number, the vector containing the gene of interest can be isolated in significant quantities for introduction into the desired plant cells.

Host cells that can be used in the practice of this invention include prokaryotes, including bacterial hosts such as *E. coli*, *S. typhimurium*, and *Serratia marcescens*. Eukaryotic hosts such as yeast or filamentous fungi may also be used in this invention. Since these hosts are also microorganisms, it will be essential to ensure that plant promoters which do not cause expression of the polypeptide in bacteria are used in the vector.

The isolated cloning vector will then be introduced into the plant cell using any convenient transformation technique as described above.

III. Regeneration and Analysis of Transformants

Following transformation, regeneration is involved to obtain a whole plant from transformed cells and the presence of structural gene (s) or "transgene(s)" in the regenerated plant is detected by assays. The seed derived from the plant is then tested for levels of preselected amino acids. Depending on the type of plant and the level of gene expression, introduction of the structural gene into the plant seed endosperm can enhance the level of preselected amino acids in an amount useful to supplement the nutritional quality of those seeds.

Using known techniques, protoplasts and cell or tissue culture can be regenerated to form whole fertile plants which carry and express the gene for a polypeptide according to this invention.

Accordingly, a highly preferred embodiment of the present invention is a transformed maize plant, the cells of which contain at least one copy of the DNA sequence of an expression cassette containing a gene encoding a polypeptide containing elevated amounts of an essential amino acid, such as HT12, BHL or ESA protein.

Techniques for regenerating plants from tissue culture, such as transformed protoplasts or callus cell lines, are known in the art. For example, see Phillips, *et al.*; Plant Cell Tissue Organ Culture; Vol. 1; p. 123; (1981); Patterson, *et al.*; Plant Sci.; Vol. 42; p. 125; (1985); Wright, *et al.*; Plant Cell Reports; Vol. 6; p. 83; (1987); and Barwale, *et al.*; Planta; Vol. 167; p. 473; (1986); each incorporated herein in its entirety by reference. The selection of an appropriate method is within the skill of the art.

Examples of the practice of the present invention detailed herein relate specifically to maize plants. However, the present invention is also applicable to other cereal plants. The expression vectors utilized herein are demonstrably capable of operation in cells of cereal plants both in tissue culture and in whole plants. The invention disclosed herein is thus operable in monocotyledonous species to transform individual plant cells and to achieve full, intact plants which can be regenerated from transformed plant cells and which express preselected polypeptides.

The introduced structural genes are expressed in the transformed plant cells and stably transmitted (somatically and sexually) to the next generation of cells produced. The vector should be capable of introducing, maintaining, and expressing a structural gene in plant cells. The structural gene is passed on to progeny by normal sexual transmission.

To confirm the presence of the structural gene (s) or "transgene(s)" in the regenerating plants, or seeds or progeny derived from the regenerated plant, a variety of assays can be performed. Such assays include Southern and Northern blotting; PCR; assays that detect the presence of a polypeptide product, e.g., by immunological means (ELISAs and Western blots) or by enzymatic function; plant part assays, such as leaf, seed or root assays; and also, by analyzing the phenotype of the whole regenerated plant.

Whereas DNA analysis techniques can be conducted using DNA isolated from any part of a plant, RNA will be expressed in the seed endosperm and hence it will be necessary to prepare RNA for analysis from these tissues.

PCR techniques can be used for detection and quantitation of RNA produced from introduced structural genes. In this application of PCR it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product.

Further information about the nature of the RNA product may be obtained by Northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an

RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and will only demonstrate the presence or absence of an RNA species.

While Southern blotting and PCR may be used to detect the structural gene in question, they do not provide information as to whether the structural gene is being expressed. Expression may be evaluated by specifically identifying the polypeptide products of the introduced structural genes or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific polypeptides may make use of physical-chemical, structural, functional, or other properties of the polypeptides. Unique physical-chemical or structural properties allow the polypeptides to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography.

The unique structures of individual polypeptides offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as Western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques.

Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Very frequently, the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms, including but not limited to, analyzing changes in the chemical composition, morphology, or physiological properties of the plant. In particular, the elevated
5 preselected amino acid content due to the expression of structural genes encoding polypeptides can be detected by amino acid analysis.

Breeding techniques useful in the present invention are well known in the art.

The present invention will be further described by reference to the following detailed examples. It is understood, however, that there are many extensions,
10 variations, and modifications on the basic theme of the present invention beyond that shown in the examples and description, which are within the spirit and scope of the present invention.

Examples

EXAMPLE 1

15 ***Construction of the HT12 gene and of other genes encoding polypeptides having an elevated level of a preselected amino acid.***

As noted above, the sequence of the HT12 gene is based on the mRNA sequence of the native *Hordeum vulgare* alpha hordothionin gene (accession number X05901, Ponz *et al.* 1986 Eur. J. Biochem. 156:131-135) modified to
20 introduce 12 lysine residues into the mature hordothionin peptide (See Rao *et al.* 1994 Protein Engineering 7(12):1485-1493, and WO 94/16078 published July 21, 1994).

The alpha hordothionin cDNA comprising the entire alpha hordothionin coding sequence is isolated by rt-PCR of mRNA from developing barley seed. Primers are

designed based upon the published alpha hordothionin sequence to amplify the gene and to introduce a NcoI site at the start (ATG) codon and a BamHI site after the stop codon of the thionin coding sequence to facilitate cloning.

Sub 17 Primers are designated as HTPCR1 (5'-
5 AGTATAAGTAAACACACCATCACACCCTTGAGGCCCTTGCTGGTGGCCATGGT
G-3') and HTPCR2 (5'-
CCTCACATCCCTTAGTGCCTAAGTTCGACGTCGGGCCCTCTAGTCGACGGATC
CA-3'). These primers are used in a PCR reaction to amplify alpha hordothionin by
conventional methods. The resulting PCR product is purified and subcloned into the
10 BamHI/NcoI digested pBSKP vector (Stratagene, LaJolla, CA) and sequenced on
both strands to confirm its identity. The clone is designated pBSKP-HT (seq. ID 1).
Primers are designed for single stranded DNA site-directed mutagenesis to
introduce 12 codons for lysine, based on the peptide structure of hordothionin 12
(Ref: Rao *et al.* 1994 Protein Engineering 7(12):1485-1493) and are designated
15 HT12mut1 (5'-AGCGGAAAATGCCCGAAAGGCTTCCCCAAATTGGC-3'),
HT12mut2 (5'-
TGCGCAGGCGTCTGCAAGTGTAAGCTGACTAGTAGCGGAAAATGC-3'),
HT12mut3 (5'-
TACAACCTTTGCAAAGTCAAAGGCGCCAAGAAGCTTTGCGCAGGCGTCTG-3'),
20 HT12mut4 (5'-
GCAAGAGTTGCTGCAAGAGTACCCTGGGAAGGAAGTGCTACAACCTTTGC-3').

Sequence analysis is used to verify the desired sequence of the resulting plasmid, designated pBSKP-HT12 (seq. ID 2).

Similarly, genes encoding other derivatives of hordothionin, as described above, (See U.S. Ser. Nos. 08/838,763 filed April 10, 1997; ~~08/824,379~~ filed March

~~26, 1997; 08/824,382~~ filed March 26, 1997; and U.S. Pat. No. 5,703,409 issued

December 30, 1997), the gene encoding enhanced soybean albumin (ESA) (See

U.S. Ser. No. 08/618,911), and genes encoding BHL and other derivatives of the

barley chymotrypsin inhibitor (See U.S. Ser. No. 08/740,682 filed November 1, 1996

and PCT/US97/20441 filed October 31, 1997) are constructed by site directed

mutagenesis from pBSKP-HT, a subclone of the soybean 2S albumin 3 gene in the

pBSKP vector (Stratagene, LaJolla, CA), and a subclone of the barley chymotrypsin

inhibitor in the pBSKP vector, respectively.

EXAMPLE 2

Construction of vectors for seed preferred expression of polypeptides having an elevated level of a preselected amino acid.

A 442bp DNA fragment containing the modified hordothionin gene encoding HT12 is isolated from plasmid pBSKP-HT12 by NcoI/BamHI restriction digestion, gel purification and is ligated between the 27 kD gamma zein promoter and 27kD gamma zein terminator of the NcoI/BamHI digested vector PHP3630. PHP 3630 is a subclone of the endosperm-preferred 27kD gamma zein gene (Genbank accession number X58197) in the pBSKP vector (Stratagene), which is modified by site directed mutagenesis by insertion of a NcoI site at the start codon (ATG) of the 27kD gamma zein coding sequence. The 27kD gamma zein coding sequence is replaced with the HT12 coding sequence. The resulting expression vector containing the chimeric gene construct gz::HT12::gz, designated as PHP8001 (Seq. ID 3), is verified by extensive restriction digest analysis and DNA sequencing.

Similarly, the 442bp DNA fragment containing the HT12 coding sequence is inserted between the globulin1 promoter and the globulin1 terminator of the embryo preferred corn globulin1 gene (Genbank accession number X59083), and between the waxy promoter and the waxy terminator of the endosperm-preferred waxy gene (Genbank accession number M24258). The globulin1 and waxy coding sequences, respectively, are replaced with the HT12 coding sequence. The resulting chimeric genes glb1::HT12::glb1, and wx::HT12::wx are designated as PHP 7999 (Seq. ID 4), and PHP 5025 (Seq. ID 5).

Sub 5
10 In a like manner, expression vectors containing genes encoding other derivatives of hordothionin (See Rao *et al.*, 1994 Protein Engineering 7(12):1485-1493, and WO 94/16078 published July 21, 1994), the gene encoding enhanced soybean albumin (ESA) (See U.S. Ser. No. 08/618,911,), and genes encoding BHL and other derivatives of the barley chymotrypsin inhibitor (See U.S. Ser. No. 08/740,682 filed November 1, 1996 and PCT/US97/20441 filed October 31, 1997) are constructed by insertion of the corresponding coding sequences between the promoter and terminator of the 27kD gamma zein gene, the globulin1 gene and the waxy gene, respectively. Resulting chimeric genes are for example gz::ESA::gz and gz::BHL::gz, designated as PHP11260 (Seq. ID 6) and as PHP11427 (Seq. ID 7), respectively.

20 The resulting expression vectors are used in conjunction with the selectable marker expression cassettes PHP3528 (enhanced CAMV::Bar::PinII) for particle bombardment transformation of maize immature embryos.

EXAMPLE 3

Preparation of Transgenic Plants

The general method of genetic transformation used to produce transgenic maize plants is mediated by bombardment of embryogenically responsive immature embryos with tungsten particles associated with DNA plasmids, said plasmids consisting of a selectable and an unselectable marker gene.

Preparation of Tissue

Immature embryos of "High Type II" are the target for particle bombardment-mediated transformation. This genotype is the F_1 of two purebred genetic lines, parent A and parent B, derived from A188 X B73. Both parents are selected for high competence of somatic embryogenesis. See Armstrong, *et al.*, "Development and Availability of Germplasm with High Type II Culture Formation Response," Maize Genetics Cooperation Newsletter, Vol. 65, pp. 92 (1991); incorporated herein in its entirety by reference.

Ears from F_1 plants are selfed or sibbed, and embryos are aseptically dissected from developing caryopses when the scutellum first becomes opaque. The proper stage occurs about 9-13 days post-pollination, and most generally about 10 days post-pollination, and depends on growth conditions. The embryos are about 0.75 to 1.5 mm long. Ears are surface sterilized with 20-50% Clorox for 30 min, followed by 3 rinses with sterile distilled water.

Immature embryos are cultured, scutellum oriented upward, on embryogenic induction medium comprised of N6 basal salts (Chu, *et al.*, "Establishment of an Efficient Medium for Anther Culture of Rice Through Comparative Experiments on the Nitrogen Sources," Scientia Sinica, (Peking), Vol. 18, pp. 659-668 (1975);

incorporated herein in its entirety by reference; Eriksson vitamins (See Eriksson, T., "Studies on the Growth Requirements and Growth Measurements of Haplopappus gracilis," Physiol. Plant, Vol. 18, pp. 976-993 (1965); incorporated herein in its entirety by reference), 0.5 mg/l thiamine HCl, 30 gm/l sucrose, 2.88 gm/l L-proline, 1
5 mg/l 2,4-dichlorophenoxyacetic acid, 2 gm/l Gelrite, and 8.5 mg/l AgNO₃.

The medium is sterilized by autoclaving at 121°C for 15 min and dispensed into 100 X 25 mm petri dishes. AgNO₃ is filter-sterilized and added to the medium after autoclaving. The tissues are cultured in complete darkness at 28°C. After about 3 to 7 days, generally about 4 days, the scutellum of the embryo has swelled
10 to about double its original size and the protuberances at the coleorhizal surface of the scutellum indicate the inception of embryogenic tissue. Up to 100% of the embryos display this response, but most commonly, the embryogenic response frequency is about 80%.

When the embryogenic response is observed, the embryos are transferred to
15 a medium comprised of induction medium modified to contain 120 gm/l sucrose. The embryos are oriented with the coleorhizal pole, the embryogenically responsive tissue, upwards from the culture medium. Ten embryos per petri dish are located in the center of a petri dish in an area about 2 cm in diameter. The embryos are maintained on this medium for 3-16 hr, preferably 4 hours, in complete darkness at
20 28°C just prior to bombardment with particles associated with plasmid DNAs containing the selectable and unselectable marker genes.

To effect particle bombardment of embryos, the particle-DNA agglomerates are accelerated using a DuPont PDS-1000 particle acceleration device. The particle-DNA agglomeration is briefly sonicated and 10 µl are deposited on

macrocarriers and the ethanol allowed to evaporate. The macrocarrier is accelerated onto a stainless-steel stopping screen by the rupture of a polymer diaphragm (rupture disk). Rupture is effected by pressurized helium. Depending on the rupture disk breaking pressure, the velocity of particle-DNA acceleration may be varied. Rupture disk pressures of 200 to 1800 psi are commonly used, with those of 650 to 1100 psi being more preferred, and about 900 psi being most highly preferred. Rupture disk breaking pressures are additive so multiple disks may be used to effect a range of rupture pressures.

Preferably, the shelf containing the plate with embryos is 5.1 cm below the bottom of the macrocarrier platform (shelf #3), but may be located at other distances. To effect particle bombardment of cultured immature embryos, a rupture disk and a macrocarrier with dried particle-DNA agglomerates are installed in the device. The He pressure delivered to the device is adjusted to 200 psi above the rupture disk breaking pressure. A petri dish with the target embryos is placed into the vacuum chamber and located in the projected path of accelerated particles. A vacuum is created in the chamber, preferably about 28 inches Hg. After operation of the device, the vacuum is released and the petri dish is removed.

Bombarded embryos remain on the osmotically adjusted medium during bombardment, and preferably for two days subsequently, although the embryos may remain on this medium for 1 to 4 days. The embryos are transferred to selection medium comprised of N6 basal salts, Eriksson vitamins, 0.5 mg/l thiamine HCl, 30 gm/l sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid, 2 gm/l Gelrite, 0.85 mg/l AgNO₃ and 3 mg/l bialaphos. Bialaphos is added filter-sterilized. The embryos are subcultured to fresh selection medium at 10 to 14 day intervals. After about 7 weeks,

embryogenic tissue, putatively transgenic for both selectable and unselected marker genes, is seen to proliferate from about 7% of the bombarded embryos. Putative transgenic tissue is rescued, and that tissue derived from individual embryos is considered to be an event and is propagated independently on selection medium.

- 5 Two cycles of clonal propagation is achieved by visual selection for the smallest contiguous fragments of organized embryogenic tissue.

For regeneration of transgenic plants, embryogenic tissue is subcultured to medium comprised of MS salts and vitamins (Murashige, T. and F. Skoog, "A revised medium for rapid growth and bio assays with tobacco tissue cultures";
10 Physiologia Plantarum; Vol. 15; pp. 473-497; 1962; incorporated herein in its entirety by reference), 100 mg/l myo-inositol, 60 gm/l sucrose, 3 gm/l Gelrite, 0.5 mg/l zeatin, 1 mg/l indole-3-acetic acid, 26.4 ng/l cis-trans-abscissic acid, and 3 mg/l bialaphos in 100 X 25 mm petri dishes and incubated in darkness at 28°C until the development of well-formed, matured somatic embryos can be visualized. This requires about 14
15 days.

Well-formed somatic embryos are opaque and cream-colored, and are comprised of an identifiable scutellum and coleoptile. The embryos are individually subcultured to germination medium comprised of MS salts and vitamins, 100 mg/l myo-inositol, 40 gm/l sucrose and 1.5 gm/l Gelrite in 100 X 25 mm petri dishes and
20 incubated under a 16 hr light: 8 hr dark photoperiod and 40 $\mu\text{Einsteins m}^{-2}\text{sec}^{-1}$ from cool-white fluorescent tubes. After about 7 days, the somatic embryos have germinated and produced a well-defined shoot and root. The individual plants are subcultured to germination medium in 125 x 25 mm glass tubes to allow further plant

development. The plants are maintained under a 16 hr light: 8 hr dark photoperiod and $40 \mu\text{Einsteins m}^{-2}\text{sec}^{-1}$ from cool-white fluorescent tubes.

After about 7 days, the plants are well-established and are transplanted to horticultural soil, hardened off, and potted into commercial greenhouse soil mixture and grown to sexual maturity in a greenhouse. An elite inbred line is used as a male to pollinate regenerated transgenic plants.

Preparation of Particles

Fifteen mg of tungsten particles (General Electric) , 0.5 to 1.8 μm , preferably 1 to 1.8 μm , and most preferably 1 μm , are added to 2 ml of concentrated nitric acid. This suspension is sonicated at 0°C for 20 min (Branson Sonifier Model 450, 40% output, constant duty cycle). Tungsten particles are pelleted by centrifugation at 10,000 rpm (Biofuge) for 1 min and the supernatant is removed. Two ml of sterile distilled water is added to the pellet and sonicate briefly to resuspend the particles. The suspension is pelleted, 1 ml of absolute ethanol is added to the pellet and sonicated briefly to resuspend the particles. Rinse, pellet, and resuspend the particles a further 2 times with sterile distilled water, and finally resuspend the particles in 2 ml of sterile distilled water. The particles are subdivided into 250 μl aliquots and stored frozen.

Preparation of particle-plasmid DNA association

The stock of tungsten particles is sonicated briefly in a water bath sonicator (Branson Sonifier Model 450, 20% output, constant duty cycle) and 50 μl is transferred to a microfuge tube. Plasmid DNA is added to the particles for a final DNA amount of 0.1 to 10 μg in 10 μl total volume, and briefly sonicated. Preferably 1 μg total DNA is used. Specifically, 5 μl of PHP8001 (gz::HT12::gz) and 5 μl of

PHP3528 (enhanced CAMV::Bar::PinII) at 0.1 $\mu\text{g}/\mu\text{l}$ in TE buffer, are added to the particle suspension. Fifty μl of sterile aqueous 2.5 M CaCl_2 are added, and the mixture is briefly sonicated and vortexed. Twenty μl of sterile aqueous 0.1M spermidine are added and the mixture is briefly sonicated and vortexed. The mixture is incubated at room temperature for 20 min with intermittent brief sonication. The particle suspension is centrifuged, and the supernatant is removed. Two hundred fifty μl of absolute ethanol is added to the pellet and briefly sonicated. The suspension is pelleted, the supernatant is removed, and 60 μl of absolute ethanol is added. The suspension is sonicated briefly before loading the particle-DNA agglomeration onto macrocarriers.

EXAMPLE 4

Analysis of seed from transgenic plants for recombinant polypeptides having an elevated level of a preselected amino acid.

Preparation of meals from corn seed

Pooled or individual dry seed harvested from transformed plants from the greenhouse or the field are prepared in one of the following ways:

- A. Seed is imbibed in sterile water overnight (16-20 hr) at 4°C. The imbibed seed is dissected into embryo, endosperm and pericarp. The embryos and endosperm are separately frozen in liquid N_2 , the pericarps are discarded. Frozen tissue is ground with a liquid N_2 chilled ceramic mortar and pestle to a fine meal. The meals are dried under vacuum and stored at -20°C or -80°C.

B. Dry whole seed is ground to a fine meal with a ball mill (Klecko), or by hand with a ceramic mortar and pestle. For analysis of endosperm only, the embryos are removed with a drill and discarded. The remaining endosperm with pericarp is ground with a ball mill or a mortar and pestle.

5 ELISA analysis

Rabbit polyclonal anti HT12 antisera are produced with synthetic HT12 (See Rao *et al. supra*) at Bethyl laboratories. An HT12 ELISA assay is developed and performed by the Analytical Biochemistry department of Pioneer Hi-Bred International, Inc., essentially as described by Harlow and Lane, Antibodies, A Laboratory Manual, Cold Springs Harbor Publication, New York (1988). Quantitative ELISA assays are first performed on pooled meals to identify positive events. Positive events are further analyzed by quantitative ELISA on individual kernels to determine the relative level of HT12 expression and transgene segregation ratio. Among 97 events tested, 59 show HT12 expression levels >1000 ppm. The highest events have HT12 expression levels at 2-5% of the total seed protein. Typical results for HT12 levels for whole kernels of wild type corn, for one event (TC2031) of corn transformed with the *gz::HT12::gz* chimeric gene, expressing HT12 in the endosperm, for one event (TC320) of corn transformed with the *wx::HT12::wx* chimeric gene, expressing HT12 in the endosperm, and for one event (TC2027) of corn transformed with the *glb1::HT12::glb1* chimeric gene, expressing HT12 in the embryo, are in Table 1.

Subtle Similarly, antisera are produced, ELISA assays are developed and assays of seed from transformed plants are performed for other derivatives of hordothionine (See Rao *et al.* 1994 Protein Engineering 7(12):1485-1493, and WO 94/16078 published July 21, 1994), for the enhanced soybean albumin (ESA) (See U.S. Ser.

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No. 08/618,911) and for BHL and other derivatives of the barley chymotrypsin inhibitor (See U.S. Ser. No. 08/740,682 filed November 1, 1996 and PCT/US97/20441 filed October 31, 1997), respectively.

Polyacrylamide gel and immuno blot analysis

5 SDS extracts of meals, molecular weight markers, and a synthetic HT12 positive control (see Rao *et al. supra*) are separated on 16.5% or 8-22% polyacrylamide gradient Tris-Tricine gels (Schagger, H. and Von Jagow, G. 1987 Anal. Biochem., 166:368). For immuno blot analysis, gels are transferred to PVDF membranes in 100 mM CAPS, pH 11; 10% methanol using a semidry blotter (Hoefer, San Francisco, CA).

10 After transfer the membrane is blocked in BLOTTO (4% dry milk in Tris-buffered saline, pH 7.5) (Johnson, D. A. , Gausch, J. W., Sportsman, J. R., and Elder, J. H. 1984, Gene Anal. Techn., 1:3). The blots are incubated with rabbit anti-HT12 (same as used for ELISA) diluted 1:2000 to 1:7500 in BLOTTO 2 hr at room temperature (22°C) or overnight at 4°C. Blots are washed 4-5X with BLOTTO, then incubated 1-2

15 hr with horseradish peroxidase-goat anti-rabbit IgG (Promega, Madison, WI) diluted 1:7500 to 1:15000 in BLOTTO. After secondary antibody, the blots are washed 3X with BLOTTO followed by 2 washes with Tris-buffered saline, pH 7.5. Blots are briefly incubated with enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL) substrate, and wrapped in plastic wrap. Reactive bands are visualized after exposure

20 to x-ray film (Kodak Biomax MR) after short exposure times ranging from 5-120 sec.

HT12 transgenic seed shows a distinctive band not seen in wild type seed at the correct molecular weight and position as judged by the HT12 positive control standard and molecular weight markers. These results indicate that the expressed HT12 prepropeptide is being correctly processed like native HT in barley. Novel polypeptide

bands co-migrating with the HT12 positive control are also observed in Coomassie stained polyacrylamide gels loaded with 10mg total extracted protein indicating substantial expression and accumulation of HT12 protein in the seed.

5 Similarly, other derivatives of hordothionin, soybean albumin, the enhanced soybean albumin (ESA), BHL and other derivatives of the barley chymotrypsin inhibitor are detected by polyacrylamide gel and immuno blot analysis.

Amino acid composition analysis

10 Meals from seed, endosperm or embryo that express a recombinant polypeptide having an elevated level of a preselected amino acid are sent to the University of Iowa Protein Structure Facility for amino acid composition analysis using standard protocols for digestion and analysis.

15 Typical results for the amino acid composition of whole kernels of wild type corn, for one event (TC2031) of corn transformed with the gz::HT12::gz chimeric gene, expressing HT12 in the endosperm, for one event (TC320) of corn transformed with the wx::HT12::wx chimeric gene, expressing HT12 in the endosperm, and for one event (TC2027) of corn transformed with the glb1::HT12::glb1 chimeric gene, expressing HT12 in the embryo, are in Table 1.

Table 1: HT12 ELISA analysis and amino acid composition of meal from whole kernels from wild type corn and from transformed corn expressing recombinant HT12.

transgene none		wx::HT12::wx	gz::HT12::gz	glb1::HT12::glb1
event	wild-type	TC320	TC2031	TC2027
ELISA				
HT 12	protein ppm 0.00	protein ppm 6200	protein ppm 8000	protein ppm 22600
AA				
	Meal % n=3	Meal % n=2	Meal % n=3	Meal % n=4
Lys	0.29	0.38	0.39	0.24
Arg	0.52	0.58	0.56	0.45
Cys	0.12	0.19	0.17	0.22

5 The results in Table 1 demonstrate corn expressing recombinant HT12 in the endosperm shows a significant increase of the preselected amino acid lysine.

Table 2: SEQUENCE INFORMATION

SEQUENCE ID	PROMOTER	GENE
Seq. 1: pBSKP-HT	None	3361-2947
Seq. 2: pBSKP-HT12	None	3361-2947
Seq. 3: PHP8001gz::HT12::gz expression vector	676-2198	2199-2612
Seq. 4: PHP7999 glb1::HT12::glb1 expression vector	3271-1834	1834-1420
Seq. 5: PHP5025 wx::HT::wx expression vector	43-1342	1343-1757
Seq. 6: PHP 11260 gz::ESA::gz expression vector	676-2198	2199-2675
Seq. 7: PHP11427 gz::BHL::gz	676-2198	2199-2450